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(54) Title: STABLY TRANSFORMED EUCARYOTIC CELLS COMPRISING A FOREIGN TRANSCRIBABLE DNA UNDER THE CONTROL OF A POL III PROMOTER

(57) Abstract

This invention provides a stably transformed eucaryotic cell comprising a pol III promoter and a foreign transcribable DNA, the foreign transcribable DNA being under the control of the pol III promoter. This invention also provides retroviral vector which comprises a chimeric t-RNA introduced into the 3' long terminal repeat (LTR) of the retroviral vector.

DESIGNATIONS OF "DE"

Until further notice, any designation of "DE" in any international application whose international filing date is prior to October 3, 1990, shall have effect in the territory of the Federal Republic of Germany with the exception of the territory of the former German Democratic Republic.

Stably Transformed Eucaryotic Cells Comprising foreign
transcribable DNA under the control of a pol III Promoter

This application is a continuation-in-part of United States
application serial number 354,171, filed May 10, 1989, the
contents of which are hereby incorporated by reference into
the present disclosure.

Throughout this application various publications are
referred by numbers within parenthesis. Full citations for
these publications may be found at the end of the
specification immediately preceding the claims.   The
disclosures of these publications, in their entireties, are
hereby incorporated by reference into this application in
order to more fully describe the state of the art to which
this invention pertains.

Background of the Invention

The potential of inhibiting the expression of specific genes
in live cells was recently demonstrated using a technique
called antisense RNA (or DNA) inhibition ("antisense
inhibition"). This technique is based on blocking the flow
of genetic information from DNA via RNA to protein, by the
introduction into the cells, of a complementary sequence (in
the form of a single stranded RNA or DNA molecule called
antisense RNA or DNA) to a portion of the target RNA.
Through nucleotide base pairing, a duplex is formed which
blocks the expression of the function or the gene encoded in
the RNA transcript through one of several possible
mechanisms: degradation of the duplex; inhibition of
translation; or other (11, 22, 37).

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Regulation of gene expression via antisense RNA was first
recognized as a naturally occurring mechanism in procaryotes
(11). The potential of using complementary sequences to
inhibit specific gene functions was demonstrated by
Paterson, et al. (28) who used complementary nucleic acids
to inhibit the translation of specific mRNA species in
vitro. However, it was the pioneering work of Zamecnik and
Stephenson (38) who first demonstrated the experimental
inhibition of the expression of specific genes in live cells
via antisense RNA. This inhibition was accomplished by the
use of a synthetic oligonucleotide, complementary to a
portion of the Rous sarcoma virus (RSV), to inhibit viral
replication in cultured cells.

Inhibition of specific genes using antisense DNA templates
(also called antisense genes) was first demonstrated by
Pestka (29) and Coleman, et al. (5) in bacteria and later by
Izant and Weintraub (16) in eucaryotic cells. This was an
important development since antisense DNA templates
("antisense templates") are capable of synthesizing
antisense RNA on a continual basis when introduced into the
eucaryotic cell and therefore have the potential to exert a
long lasting effect on the cell and its progeny.

It is important to distinguish between transient antisense
and stable antisense inhibition protocols because each of
these protocol achieve different objectives and employ
different technologies. Transient antisense inhibition, as
the name implies, results in the creation of a temporary
state of inhibition in the cell. The use of antisense
oligonucleotides, microinjection of antisense RNA or

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transient DNA transfection protocols, and use of SV40 based
vectors are examples of protocols by which transient
antisense inhibition is accomplished (22).

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On the other hand, stable antisense RNA inhibition involves
the permanent genetic alteration of the cell, achieved by
the introduction of antisense templates which persist in the
cell and its progeny, by synthesizing antisense RNA on a
10   continual basis.   The stable introduction of antisense
templates into the cell can be accomplished by using any of
several gene transfer techniques. Such techniques for the
alteration of cells may involve, but are not limited to: (i)
physical methods such as $CaPO_4$ mediated DNA transfection;
15   (ii) electroporation; or (iii) use of viral vectors such as
retroviral vectors.   These methods can produce stably
altered cells by the insertion of the antisense DNA into the
cell chromosome or, via use of viral vectors, such as bovine
papilloma virus or Epstein-Barr virus, introduction of the
20   antisense templates will persist in the cell as freely
replicating episomes (22).

Stable antisense inhibition

25   The stable form of antisense RNA inhibition has an enduring
effect which creates a constant state of gene inhibition in
the host cell and its progeny. It therefore provides a
distinct advantage over the use of the transient protocol.

30   A stable antisense inhibition protocol requires the design
of a DNA template which upon transfer to the cell is capable
of synthesizing adequate levels of antisense RNA to inhibit

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the expression of the target gene. To accomplish this objective, the DNA template must encode an efficient transcriptional unit. A eucaryotic transcriptional unit comprises: (i) a promoter to initiate RNA transcription; (ii) the template of the RNA transcript; and (iii) a third region signalling RNA transcription termination. Three distinct RNA polymerases are known to exists in eucaryotic cells: (a) RNA polymerase I (pol I) which transcribes ribosomal genes; (b) RNA polymerase II (pol II) which is responsible for the expression of the protein coding cellular genes; and (c) RNA polymerase III (pol III) which transcribes the 5S and t-RNA genes.

A stable antisense inhibition protocol also requires an excess of RNA transcripts because antisense RNA inhibition requires the formation of stable duplexes between the antisense RNA and the target RNA. Indeed, several studies have shown that an apparent 30-100 fold excess of antisense RNA is required to obtain a 10-20 fold reduction in gene expression (16, 17, 20 and 26). However, other studies have shown that in some experimental systems a more moderate excess of 3-10 fold may lead to an observable inhibition (4, 21, 25 and reviewed in reference 22).

Therefore, the effectiveness of stable and transient antisense RNA inhibition is determined by the ratio of antisense RNA to sense RNA, which in turn is determined by the efficiency of antisense RNA synthesis from the corresponding DNA template.

The development of effective stable antisense inhibition

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protocols in eucaryotic cells is hampered by the lack of efficient antisense templates that are capable of synthesizing sufficient levels of antisense RNA. The nature of the promoter and its polymerase are of pivotal importance in the design of efficient antisense templates which will produce high levels of antisense RNA in cells stably transduced with the antisense template. So far, pol II based promoters have been used exclusively to generate antisense transcripts in stable antisense inhibition protocols (22).

Pol III based transcriptional units

In an attempt to improve antisense RNA synthesis using stable gene transfer protocols, the use of pol III promoters to drive the expression of antisense RNA can be considered. The underlying rationale for the use of pol III promoters is that they can generate substantially higher levels of RNA transcripts in cells as compared to pol II promoters. For example, it is estimated that in a eucaryotic cell there are about 6×10^7 t-RNA molecules and 7×10^5 mRNA molecules, i.e, about 100 fold more pol III transcripts of this class than total pol II transcripts (39). Since there are about 100 active t-RNA genes per cell, each t-RNA gene will generate on the average RNA transcripts equal in number to total pol II transcripts. Since an abundant pol II gene transcript represents about 1% of total mRNA while an average pol II transcript represents about 0.01% of total mRNA, a t-RNA (pol III) based transcriptional unit may be able to generate 100 fold to 10,000 fold more RNA than a pol II based transcriptional unit.

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Several reports have described the use of pol III promoters to express RNA in eucaryotic cells. Lewis and Manley (23) and Sisodia (34) have fused the Adenovirus VA-1 promoter to various DNA sequences (the herpes TK gene globin and tubulin) and used transfection protocols to transfer the resulting DNA constructs into cultured cells which resulted in transient synthesis of RNA in the transduced cell. De la Pena and Zasloff (6) have expressed a t-RNA-Herpes TK fusion DNA construct upon microinjection into frog oocytes. Jennings and Molloy have constructed an antisense RNA template by fusing the VA-1 gene promoter to a DNA fragment derived from SV40 based vector which also resulted in transient expression of antisense RNA and limited inhibition of the target gene (18).

Common to the above cited studies is that the pol III based DNA templates were introduced into cells using a gene transfer procedure which led only to transient synthesis of pol III driven RNA transcripts in the cell. Furthermore, since the number of active DNA templates present in the transiently transduced cells cannot be determined, the efficiency of pol III based transcription was not and could not have been determined.

It is the objective of this invention to design improved DNA templates which can be stably transferred into the eucaryotic cell and which are capable of synthesizing large quantities of antisense RNA, RNA, or gene product.

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Summary of the Invention

This invention provides a stably transformed eucaryotic cell
comprising a pol III promoter and a foreign transcribable
DNA, the foreign transcribable DNA being under the control
of the pol III promoter.

This invention also provides a retroviral vector which
comprises a chimeric t-RNA introduced into the 3' long
terminal repeat (LTR) of the retroviral vector.

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Description of the Figures

Figure 1: Structure of a prototype t-RNA, a human t-RNAi^met derivative and a chimeric t-RNA gene.

Figure 1A shows the structure of a prototype t-RNA gene. A t-RNA gene is 85-95 base pairs long. Two regions designated A and B encode the promoter which directs the initiation of RNA transcription to generate the primary transcript. Termination of transcription is specified by a run of four or more T residues on the sense strand. Arrow indicates that transcription usually terminate after the third T. The primary t-RNA transcript is further processed to remove sequences both from the 5' end and the 3' end as shown, to generate the mature t-RNA transcript. (Additional modifications including addition of CCA and base modifications are not shown). For additional information see the review by Geiduschek, 1988 (9).

Figure 1B shows the structure of a human tRNAi^met derivative 3-5. 3-5 was derived from a cloned human t-RNA gene by deleting 19bp from the 3' end of the gene (1). The truncated gene can be transcribed if a termination signal is provided, however, no processing of the 3' end of the primary transcript takes place.

Figure 1C shows a chimeric t-RNA gene. A foreign sequence is fused to the 3' end of t-RNAi^met 3-5 and a termination signal also is added. Transcription results in the formation of a chimeric RNA species consisting of the t-RNA transcript fused to the foreign sequence.

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Figure 2: Sequence of three DNA fragments fused to t-RNAi^{met}
3-5.

5   Figure 2 shows that the DNA fragments A and B correspond to
     sequence found in an HIV isolate, right to left, nucleotides
     530 to 559 and 5960 to 5989, respectively (33).   DNA
     fragment C corresponds to a sequence found in M-MuLV,
     nucleotides 1645 to 1674 (31). Additional sequences at the
10   5' end of each DNA fragment generate a Sac II "sticky end"
     as indicated.   At the 3' end of each fragment, additional
     nucleotides are present to generate a t-RNA transcription
     termination signal and a MluI "sticky ends", as indicated.

15   Figure 3:   Structure of a retroviral vector containing a
     chimeric t-RNA gene.

     N2 is a retroviral vector derived from M-MuLV, a murine
     retrovirus, which was previously described (2).   The N2
20   vector was first modified by insertion of a 52 bp long
     polylinker sequence into the NheI site present in the 3'LTR.
     The polylinker sequence contains five restriction sites
     which are unique to the N2 plasmid: ApaI, Bgl II, Sna BI,
     Sac II, and MluI.   (14).   The t-RNA containing DNA fragment
25   encoded in plasmid 3-5 (as described by Adeniyi-Jones (1))
     was excised with Stu I and Bam HI, treated with Klenow
     fragment to generate blunt ends and cloned into the Sna BI
     site of the modified N2 vector.   The three DNA fragments
     shown in Figure 2 were cloned into the Sac II and Mu I sites
30   of the polylinker in the N2 vector.   The sequences as shown
     in Figure 2 fused to the 3' end of 3-5 will generate fusion
     RNA   transcripts   in   which   the   foreign   sequenc   is

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complementary to HIV RNA (A and B) or M-MuLV (C). N2 vector
DNA containing the chimeric t-RNA was converted to
corresponding virus using established procedures as
described in text (14, 27). Figure 3 also shows that in the
infected cell the chimeric t-RNA is duplicated and is
present in both LTR's (14). LTR - long terminal repeat;
Neo-Neomycin resistance gene; 3-5- tRNAi^{met} derivative
described in Figure 1B; seq - DNA fragments described in
Figure 2; T-Transcription termination signal shown in Figure
2.

Figure 4: RNA blot analysis of cells infected with antisense
vectors containing chimeric t-RNAs.

Virus containing chimeric t-RNAs was generated by
transfection of packaging cells, PA317, according to
established procedures (12, 27). Total cellular RNA was
isolated, subjected to electrophoresis in 8% urea-
polyacrylamide gels, blotted to nitrocellulose filters and
hybridized with a human t-RNAi^{met} probe. This probe detects
both human and mouse t-RNAi^{met} RNA species (13) as well as
the chimeric t-RNA transcripts. Panels A, B and C show that
two RNA transcripts are detected in uninfected cells (NIH
3T3 panel A and B; HUT 78, panel C). The larger species
represents the primary RNA transcript in mouse cells and the
shorter species is the mature t-RNA. (Note that the primary
transcript in mouse cells is longer than that of the human
cells but the size of the mature t-RNA is identical). Cells
harboring a chimeric t-RNA contain an additional RNA species
which is detected with the t-RNAi^{met} probe. This RNA species
corresponds in size to the predicted size of the transcripts

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expressed from the corresponding templates (see Figures 2 and 3), and it is of the same size in both human and mouse cells.

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Panel A shows virus corresponding to DNA construct DCT5A and DCT5B which were used to infect NIH 3T3 cells. G418 resistant colonies were isolated and then pooled for RNA analysis. The variation in intensity of the chimeric t-RNA 10 band is due to the variation in the fraction of cells harboring the correct vector DNA.

Panel B shows NIH 3T3 cells infected with virus corresponding to DCT5C. G418 resistant colonies were 15 isolated (C1-C4) and analyzed independently.

Panel C shows HUT 78 cells infected with DCT5A virus G418 resistant colonies which were isolated in soft agar (A1-A3) and analyzed independently.

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Figure 5: Inhibition of M-MuLV replication in NIH 3T3 cells harboring antisense vectors.

Cloned cell lines (10^6 cells per 6 cm plate) harboring the 25 M-MuLV specific antisense vector DCT5C 1, 2 and 4 - (see RNA analysis, Figure 4B) and the parent vector DCT5 without the antisense sequences but containing the 3-5 t-RNAi^{met} derivative (DCT5 1, 2) were infected with M-MuLV at a M.O.I. of about 2 and secretion of virus was determined 3 days post 30 infection by measuring R.T. activity in the medium (10).

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Figure 6:   Inhibition of viral spread in a culture of NIH
3T3 cells harboring an antisense vector.

5   NIH 3T3 cells and a clonaly derived cell line harboring an
antisense vector (DCT5C-2, see Figures 4 and 5) were
infected with M-MuLV at a M.O.I. of 0.002 (10^6 cells per 6
cm plate).   Cells were grown to semiconfluency and split
1:20.   At times indicated (days 4, 6, 8 and 11) cells were
10   removed from the plate by light tripsination and the
fraction of cells harboring virus was measured by
determining the presence of viral specific envelope on the
cell surface using immunofluorescence staining and FACS
analysis.   Briefly, the trypsinized cells were reacted with
15   a M-MuLV envelope specific monoclonal antibody followed by
FITC labelled goat antimouse antibody and sorted on a FACS
machine. 10^1 fluorescence units were arbitrarily chosen to
distinguish (gate) between negative and positive cells. The
upper two panels show that in a culture of NIH 3T3 cells
20   chronically infected with M-MuLV 91.8% cells score as
positively infected cells and in a culture of uninfected NIH
3T3 cells only 1.8 score as positive, i.e. constituting the
experimental background of this procedure. The lower left
panels show that in cultures infected with a low
25   multiplicity of virus (M.O.I. 0.002, i.e. one cell in 5000),
virus infection spreads through the culture and after 8 and
11 days, 80% and 90% of cells are infected respectively. In
contrast, the panels on the right show that spread of virus
in cultures containing antisense vector is significantly
30   inhibited, reaching about 3% and 11% after 8 and 11 days
respectively.

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Figure 7:   Inhibition of HIV replication in HUT 78 cells
harboring antisense vectors.

5   HUT 78 cells were infected with DCT5A and DCT5B virus and a
       control virus (DCA - in which the human ADA minigene was
       inserted into the 3' LTR of the N2 vector) (14). Two days
       post-infection, the cells were plated in soft agar in the
       presence of 0.7 mg/ml G418. Resistant colonies appeared 10-
10   14 days later in cultures infected with the various vectors.
       Independent colonies were isolated and expanded to cell
       lines for further use.   Expression of the chimeric t-RNA
       transcripts was determined in the DCT5A containing cell
       lines (A1-A3) as shown in Figure 4C, but was not determined
15   for the three DCA containing cell lines (control) or for the
       DCT5B derived cell lines (B1-B4).

       Each cell line was infected with HIV (the virus strain used
       was ARV-2 (31), 10^5 cells per ml of 1:10 dilution of virus)
20   and cells were split 1:5 every three to four days. Presence
       of HIV in the cell cultures was determined 14 days post HIV
       infection by measuring the R.T. activity in the various cell
       lines (average of the three control cell lines is taken as
       zero inhibition) is indicated for each cell line harboring
25   an antisense vector (A1-A3 and B1-B4).   In four cell lines
       R.T. activity was undetectable (100% inhibition), in two
       cell lines, A2 and A3, a low level of R.T activity was
       detected (92% and 82% inhibition, respectively) and in one
       cell line B3, HIV replication was not inhibited.   Since RNA
30   analysis was not performed from DCT5B containing cell lines,
       the most plausible explanation is that this cell line did
       not contain or did not express (sufficient levels) of the

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antisense RNA.

Figure 8 shows the 60 base pair oligonucleotide encoding the
TAR sequence of the Human Immunodeficiency Virus (HIV).

Figure 9 shows inhibition of R.T. activity in TAR "decoy"
containing cells. In this experiment, the ability of HIV to
replicate in cells expressing TAR was compared to cells
which were treated in parallel to contain a similar vector
not expressing TAR.

Figure 10 shows inhibition of R.T. activity in TAR "decoy"
containing cells up to 24 days post infection.

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Detailed Description of the Invention

5   This invention provides a stably transformed eucaryotic cell, i.e., a cell into which a foreign DNA or RNA fragment was introduced so that the foreign DNA or RNA, or its transcript, or reverse transcript is maintained in the progeny over many generations, comprising a pol III promoter and a foreign transcribable DNA, the foreign transcribable
10 DNA being under the control of the pol III promoter.

The foreign transcribable DNA may be virtually any DNA capable of being transcribed into RNA, regardless of whether such RNA is subsequently translated into a polypeptide for
15 example, the transcribable DNA may encode an RNA capable of acting as a false primer, i.e. a primer for the initiation of reverse transcription; and ribozyme, i.e. an enzyme made of RNA, not protein; an antisense RNA; or an mRNA, including a polypeptide translatable therefrom; or a viral regulatory
20 sequence such as the HIV regulatory sequence designated TAR. The pol III promoter may be any promoter recognized by a pol III RNA polymerase. Examples of pol III promoters useful in the practice of this invention include, but are not limited to, t-RNA pol III promoter, such as human, plant or animal
25 pol III promoters or mutants or derivations including chimeric derivatives thereof, e.g. a human t-RNAi^{met} or the 3-5 derivative thereof. The foreign transcribable DNA under the control of the pol III promoter may be introduced into the cell by any gene transfer method. Such methods may
30 include, but are not limited to, the use of gene transfer vectors, CAPO$_4$ mediated DNA transfection or electroporation.

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In one embodiment, the foreign transcribable DNA encodes
antisense RNA which is complementary to a segment of an RNA
encoded by a pathogen, such as a retrovirus, e.g. the Human
Immunodeficiency Virus (HIV) or a bacteria or a parasite.
Alternatively, the transcribable DNA may encode an HIV
regulatory sequence, such as the regulatory sequence
designated TAR to which the HIV regulatory protein
designated tat binds, to activate HIV transcription, or the
REV recognition signal of HIV.

The foreign transcribable DNA may encode a molecule which
inhibits the expression of a gene within the cell such as an
endogenous gene or a foreign gene, e.g. a viral or
retroviral gene present within the cell. For example, the
foreign transcribable DNA may encode a recognition sequence
of a regulator of gene expression which acts through binding
to a DNA molecule, an RNA molecule, or a regulatory protein,
within the cell.

The eucaryotic cell useful in the practice of this invention
may be a plant or animal cell, such as a mammalian cell,
e.g. a human cell, or chicken cell. The mammalian and human
cells may comprise but are not limited to haematopoietic
stem cells.

The invention also provides a stably transformed eucaryotic
cell comprising a pol III promoter and a foreign
transcribable DNA, the transcribable DNA being under the
control of the pol III promoter, wherein the pol III
promoter and the foreign transcribable DNA are present in a
gene transfer vector. The gene transfer vector may be a

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retroviral vector. The gene transfer retroviral vector also may comprise a chimeric t-RNA introduced into the 3' long terminal repeat (LTR) region of the retroviral vector.

The retroviral vectors useful in the practice of this invention are, but not limited to, the murine retrovirus designated M-MuLV; the retrovirus designated N2; and the double copy vectors designated DCT5A, DCT5B and DCT5C. Additionally, in accordance with the practice of this invention, the foreign DNA molecule may be under the control of a t-RNA termination signal, and the termination signal having removed therein the 3' end processing DNA sequences.

The stably transformed eucaryotic cell may comprise two or more pol III promoters and two or more foreign transcribable DNAs, each under the control of one of the pol III promoters. The two or more pol III promoters may be contained within a single gene transfer vector, e.g., a retroviral vector.

This invention further provides a retroviral vector which comprises a chimeric t-RNA introduced into the 3' long terminal repeat (LTR) of the retroviral vector. The chimeric t-RNA may comprise a pol III promoter and a foreign transcribable DNA, the transcribable DNA being under the control of the pol III promoter. The retroviral vector also may comprise two or more pol III promoters and two or more transcribable DNAs, each under the control of one of the pol III promoters.

The foreign transcribable DNA may be virtually any DNA

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capable of being transcribed into RNA, regardless of whether
such RNA is subsequently translated into a polypeptide, for
example, the transcribable DNA may encode an RNA capable of
5   acting as a false primer, i.e. a primer for initiation of
reverse transcription; a ribozyme, i.e. an enzyme made of
RNA, not protein; an antisense RNA; or an mRNA, including a
polypeptide translatable therefrom; or a viral regulatory
sequence such as the HIV regulatory sequence designated TAR.
10   The pol III promoter may be any promoter recognized by a pol
III RNA polymerase. Examples of pol III promoters useful in
the practice of this invention include, but are not limited
to, t-RNA pol III promoter, such as human, plant or animal
pol III promoter or mutant or derivative including chimeric
15   derivatives thereof, e.g. a human t-RNAi^{met} or the 3-5
derivative thereof.

In one embodiment the foreign transcribable DNA encodes
antisense RNA which is complementary to a segment of an RNA
20   encoded by a pathogen, such as a retrovirus, e.g. the Human
Immunodeficiency Virus (HIV) or a bacteria or a parasite.
Alternatively, the transcribable DNA may encode an HIV
regulatory sequence, e.g., the TAR sequence, or the REV
recognition signal of HIV.
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The foreign transcribable DNA may encode a molecule which
inhibits the expression of a gene within the cell such as an
endogenous gene or a foreign gene, e.g. a viral or
retroviral gene present within the cell. For example, the
30   foreign transcribable DNA may encode a recognition sequence
of a regulator of gene expression which acts through binding
to a DNA, an RNA molecule, or a regulatory protein, within

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the cell.

The gene transfer retroviral vector also may comprise a
chimeric t-RNA introduced into the 3' long terminal repeat
(LTR) region of the retroviral vector. The retroviral
vectors useful in the practice of this invention are, but
are not limited to, the murine retrovirus designated M-MuLV;
the retrovirus designated N2; and the double copy vectors
designated DCT5A, DCT5B and DCT5C. Additionally, in
accordance with the practice of this invention, the foreign
DNA molecule may be under the control of a t-RNA termination
signal, and the termination signal having removed therein
the 3' end processing DNA sequences.

This invention further provides stably transformed animal
cells; plant cells; mammalian cells, for example, mammalian
and human hematopoietic stem cells; and chicken cells, each
of which contain the pol III promoter and a foreign
transcribable DNA, wherein the transcribable DNA is under
the control of the pol III promoter, as described above.
These stably transformed cells may be contained within
transgenic animals, transgenic plants, transgenic mammals
and transgenic chickens, respectively.

The transgenic animal, plant, mammal or chicken described
above may contain a foreign transcribable DNA which encodes
for an RNA molecule which is complementary to a segment of
an RNA encoded by a pathogen. The pathogen may be, but is
not limited to, a retrovirus such as HIV, or a bacteria or
a parasite.

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A vaccine useful for immunizing a patient against HIV infection is provided by this invention which comprises an effective amount of the retroviral vector described hereinabove wherein the foreign transcribable DNA encodes an HIV regulatory sequence, for example, the HIV regulatory sequence designated TAR and a suitable carrier. Suitable carriers useful in the practice of this invention include, but are not limited to any pharmaceutically acceptable carrier such as sterile saline, phosphate buffered saline or an emulsion.

This invention also provides a method of producing a foreign RNA which comprises culturing the stably transformed cells described above, under conditions permitting transcription of the transcribable DNA, thereby producing the foreign RNA. This method further provides recovering the foreign RNA molecule so produced. The foreign RNA molecule may comprise, but is not limited to antisense RNA, mRNA or unprocessed RNA.

This invention further provides a method of producing a polypeptide comprising culturing the stably transformed eucaryotic cells described above under conditions permitting transcription of the transcribable DNA into RNA and translation of the RNA into a polypeptide. In one embodiment of this invention, the polypeptide so produced is recovered.

This invention also provides a method of treating an Acquired Immunodeficiency Syndrome (AIDS) patient or preventing HIV infection in a patient which comprises

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administering to the patient a retroviral vector as
described hereinabove wherein the foreign transcribable DNA
encodes for an antisense RNA molecule complementary to a
5   segment encoded by HIV or a retroviral vector wherein the
foreign transcribable DNA encodes an HIV regulatory sequence
such as the sequence designated TAR. Suitable methods of
administering the retroviral vector in pharmaceutical form
are well known to those of ordinary skill in the art, and
10 include but are not limited to administration of the
retroviral vector in a pharmaceutically acceptable carrier.
Suitable pharmaceutical carriers are described hereinabove.
Suitable methods of administration include, but are not
limited to administration orally, intravenously or
15 parenterally. Administration of the vector must be in dose
and in such a form such that the vector is transduced into
the cell, so that the foreign DNA sequence is transcribed in
an amount effective to inhibit HIV infection and/or
replication.
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A method of intracellularly immunizing a patient against HIV
infection is also provided which comprises removing
haematopoietic stem cells from the patient and infecting the
removed stem cells with an effective amount of a retroviral
25 vector described hereinabove wherein the foreign
transcribable DNA encodes for an HIV regulatory sequence,
such as the regulatory sequence designated TAR. The
infected cells are then administered back into the patient,
i.e. into the patient's bone marrow, thereby intracellularly
30 immunizing the patient against HIV infection. For the
purposes of this invention, intracellular immunization means
prophylactixis as well as treatment of an infection.

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Materials and Methods

The t-RNA transcriptional unit

Figure 1A shows the structure of a prototype mammalian t-RNA gene (ranging in size between 95-105 bp). In contrast to protein encoding genes (transcribed by pol III) the t-RNA promoter is encoded within the t-RNA gene itself, in two regions shown in Figure 1 as Box A and B. Termination of transcription is specified by a sequence present at the 3' end of the t-RNA gene (see Figure 1) which includes a run of 4 of more T nucleotides bracketed by 2 or more G or C nucleotides (on the sense strand of the DNA). Transcription of the t-RNA gene terminates in the second or third T. The RNA transcribed of the t-RNA gene, the primary transcript, is further processed resulting in the removal of sequences from the 5' and 3' end of the primary transcript, as shown in Figure 1 (9).

Human t-RNAi^{met} and the 3-5 derivative

The human genome contains 10-12 t-RNAi^{met} genes which are highly conserved among eucaryotes (32) (the human and mouse t-RNAi^{met} genes are identical and do not cross-hybridize to other cellular t-RNAs (13)) and are responsible for 10-15% of total t-RNA synthesized in mammalian cells. As calculated above, expression of RNA from one t-RNAi^{met} gene can be equal to or exceed the amount of total mRNA (pol II based) transcripts expressed in the cell. Figure 1B shows the structure of a t-RNAi^{met} derivative, called 3-5 which was us d in the studies described below. 3-5 contains a

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deletion of 19 nucleotides from the 3' end of the t-RNA gene which has effectively eliminated the 3' processing signal, i.e. the removal of the sequences 3' to the mature t-RNA (1). The studies of Adeniyi-Jones (1) strongly suggests that insertion of foreign sequences between the end of the 3-5 t-RNA derivative and a termination signal will result in the synthesis of a chimeric RNA in which the bulk of the t-RNA transcript will remain fused to the foreign RNA sequence. This is illustrated in Figure 1C.

Other t-RNA derivatives, as well as other pol III, promoters (such as ribosomal 5S or adenovirus VAI promoters) can be used to affect the processing and/or cellular localization of the foreign RNA transcript. For example, by using the t-RNAi[met] derivative described by Adeniyi-Jones (1) which is called 3-2, the foreign RNA transcript will be removed and separated from the t-RNA moiety. By using t-RNAi[met] mutants as described by Tobian, et al. (35) it may be possible to direct the RNA transcripts into the nucleus or cytoplasm of the cell.

Construction of chimeric t-RNA genes

A chimeric t-RNA is a transcriptional unit formed by fusion of a foreign DNA sequence to the 3' end of a t-RNA gene or a portion of a t-RNA gene, which encodes the t-RNA promoter but lacks a transcriptional termination sequence, and a third DNA fragment fused to the 3' end of the foreign sequence which encodes a t-RNA transcription termination signal.

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Using standard recombinant DNA techniques, three chimeric 3-
5 t-RNA genes were generated by fusing 29 and 30 bp long DNA
fragments to the 3' end of 3-5, followed by a short sequence
5   to specify termination of transcription (Figure 1C). The
sequences of the three DNA fragments are shown in Figure 2.
The question addressed in these studies is whether stable
gene transfer of the chimeric t-RNA genes into cultured
cells will result in the synthesis of the corresponding RNA.
10

Cloning into DC retroviral vector

A retroviral vector was used to introduce the chimeric t-RNA
genes into cultured mouse fibroblast cells. Figure 3 shows
15   the structure of the retroviral vector used, the method of
cloning and the essential features of this particular
vector. The retroviral vector is a double copy (DC) vector
called N2A and is derived from M-MuLV, a murine retrovirus
(12). The chimeric t-RNA genes were cloned into the 3' LTR
20   to generate vector constructs DCT5A, DCT5B and DCT5C. Using
established procedures, the vector DNA was converted to
corresponding virus and this virus was used to infect NIH
3T3 cells, an established mouse fibroblast cell line. In
the infected cell it is expected that the chimeric t-RNA
25   inserts will be duplicated and will appear in both 5' and 3'
LTR as shown in Figure 3, a feature that may facilitate the
expression of the hybrid t-RNA genes (14).

Analysis of cells infected with the chimeric t-RNA vectors
30

The three t-RNA based vectors described above and shown in
Figure 2 and 3 were converted to corresponding virus using

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established procedures (14, 27). Briefly, DCT5A and DCT5B
vector DNAs were transfected into PA317, an amphotropic
packaging cell line (27), G418 resistant colonies were
5   pooled and supernatant was used as a source of virus to
infect NIH 3T3 cells. Vector infected NIH 3T3 cells were
selected in G418 and pooled colonies were analyzed. DCT5C
virus was prepared by transient transfection of PA317 cells;
infection of NIH 3T3 cells with supernatant collected 48
10 hours post transfection; G418 selection; and isolation of
individual G418 resistant colonies were analyzed as
described below. The structure of the vector DNA integrated
in the NIH 3T3 cell chromosome was determined using known
DNA blotting procedures and was shown to be intact (not
15 shown). [As shown in Figure 3 and discussed in an article
by Hantzopoulos, et al. (14), using DC vector, the chimeric
t-RNA is duplicated in the infected cell.]

RNA blot analysis was performed to determine whether the
20 cells harboring the chimeric t-RNA express the corresponding
RNA in NIH 3T3 cells. Total cellular RNA was subjected to
electrophoresis in 8% acrylamide-urea gels, blotted to nylon
filters and hybridized with a ^{32}P-labelled t-RNAi^{met} specific
probe. The results of this analysis are shown in Figure 4A
25 and 4B. The t-RNA probe detects two RNA species in
uninfected NIH 3T3 cells in the size range of 70-90
nucleotides which represent the mature t-RNAi^{met} and its
unprocessed form. In cells infected with the t-RNA fusion
genes an additional species is detected which corresponds in
30 size to the chimeric RNA transcripts in which the t-RNA is
fused to the foreign sequence.

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Judging from the intensity of hybridization, the chimeric transcripts represent between 5% - 25% of total t-RNAi^{met} synthesized in the cell, suggesting that they are expressed very efficiently. [It is possible to estimate that if t-RNAi^{met} represent 10-15% of total t-RNA, and there are 10-12 t-RNA genes per genome and t-RNAs are 100 fold more abundant than the mRNA i.e., pol II transcripts, then the chimeric t-RNA present in the cell equals (within an order of magnitude) the total number of polyA+ transcripts. This would mean that the chimeric t-RNA carrying a foreign sequence is 100 fold to 10,000 fold more abundant than individual pol II transcripts. Expression of the chimeric t-RNA genes is not limited to the mouse fibroblast cell line NIH 3T3. Virus supernatant corresponding to DCT5A and DCT5B was used to infect HUT 78 cells, a human T-lymphoid cells line and individual clones isolated by G418 selection were analyzed for RNA expression. As shown in Figure 4C, the chimeric t-RNA genes are equally active in this human cell line.

Construction of a vector containing the HIV - TAR sequence

Construction of the N2A vector was described previously. The 3-5 tRNAi^{met} gene was then cloned into the Sna B1 site of the N2A vector such that transcription occurs parallel to LTR initiated transcription.

The pol III termination sequence "Ter" was cloned between the Sac II-MluI sites of the N2A polylinker. This oligonucleotide sequence contains a Bam HI restriction site. Previously, other Bam HI sites were removed from th v ctor

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using techniques known to those of ordinary skill in the
art.

5   The 63 base pair TAR oligonucleotide (see Figure 8) was then
cloned between the Sac II - Bam HI sites from the "Ter"
oligonucleotide upstream of the pol III termination
sequence.   This TAR sequence is derived from the ARV-Z
strain of HIV-I (31).

10

Inhibition of M-MuLV replication in cells harboring chimeric t-RNA antisense templates

DCT5C contains a chimeric t-RNA gene fused to a 29
15   nucleotide long sequence which is complementary to a portion
of M-MuLV.   M-MuLV is a prototype murine retrovirus which
replicates efficiently in NIH 3T3 cells expressing high
levels of viral RNA in the cells reaching 1-5% of total
polyA+ RNA (7, 36).   The ability of DCT5C to inhibit M-MuLV
20   replication represents a stringent test as to the potential
of this new approach to inhibit the expression of genes and
protect cells from the replication of viruses.

In the experiment shown in Figure 5, three NIH 3T3 derived
25   clones harboring the DCT5C vector and two clones containing
the parent vector, DCT5 (which does not contain the
antisense sequence) were infected with M-MuLV at a
multiplicity of infection (M.O.I.) of about 2.   The ability
of the cells to support the replication of M-MuLV was
30   determined by measuring the secretion of virus from the
cells (determined by the appearance of reverse transcriptase
(R.T.) activity in the supernatant of infected cells).   As

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shown in Figure 5, in cells harboring the antisense
templates M-MuLV replication was inhibited 70%-95%.

5   In a second experiment to test the ability of the antisense
vectors to inhibit the spread of M-MuLV in culture, the
above described cell lines were infected at a M.O.I. of
0.002 and the spread of M-MuLV was monitored by FACS
analysis, to determine the fraction of cells infected with
10   M-MuLV. As shown in Figure 6, presence of M-MuLV specific
antisense templates in cells leads to significant inhibition
in the ability of M-MuLV to spread in the culture.

The experiments described in this section shows that: (a)
15   chimeric t-RNA based transcriptional units can be stably
introduced into eucaryotic cells resulting in high levels of
corresponding RNA expression; and (b) a M-MuLV specific
antisense template fused to the t-RNA promoter results in
the efficient inhibition of M-MuLV replication in the
20   corresponding cells. Since M-MuLV is a very efficient
transcriptional unit, the experiments shown in Figures 5 and
6 testify to the potency of a pol III based transcriptional
unit.

25   **Inhibition of HIV replication in cells harboring chimeric t-
RNA antisense templates**

DCT5A and DCT5B contain a chimeric t-RNA gene fused to 30
base-pair long nucleotide sequences, which upon
30   transcription yield RNA species which are complementary to
portions of the HIV genome (see Figures 2 and 3). The
ability of the two vectors, DCT5A and DCT5B, to inhibit the

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replication of HIV in susceptible human lymphoid cells was
tested and the results are shown in Figure 7.

5   Virus corresponding to DCT5A and DCT5B was used to infect
        HUT 78 cells (a human cutaneous T cell lymphoma derived cell
        line) and individually infected cells were cloned in soft
        agar in the presence of G418. Independently derived clones
        were tested for their ability to support the replication of
10   HIV. As a control, HUT 78 cells were infected with a virus
        derived from a similar vector in which an unrelated sequence
        (the human adenosine deaminase minigene) was introduced into
        the 3'LTR of the N2 derived vector. The ability of HIV to
        replicate in HUT 78 cells containing the antisense vectors
15   and the control vector was determined by measuring the
        appearance of R.T. activity in the medium. Figure 7 shows
        the result of such an experiment. As shown in Figure 7,
        replication of HIV cell lines harboring the DCT5A vector
        (A1-A3) is inhibited between the three cell lines and this
20   correlates to the amount of antisense RNA present in the
        cell as can be seen in Figure 4C. Replication of HIV in the
        three cell lines harboring the vector DCT5B is virtually
        shut down (Figures 7, B1, B2 and B4). In one cell line, B3,
        HIV replication was not affected. The reason for that is
25   not known. The ability of HIV to replicate in the HUT 78
        derived cell lines was also measured using in site
        immunofluorescence (IFA) and the results of this experiment
        were consistent with R.T. analysis (not shown). For
        example, after 14 days in culture, 40-80% of the cells in
30   the three control cell lines scored positive while no
        positive cells were detected in cell lines A1, B1, B2 and B4
        (in a field containing about 1000 cells) and only few

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positive cells were present in A2 and A3 cells, while B3
cells were indistinguishable from the control.

5   In summary, the experiments described in this section show
     that the stable transfer into cells of DNA constructs which
     consist of chimeric t-RNAs fused to HIV specific antisense
     templates, inhibit HIV replication. In other words, the
     antisense vector containing cells are protected from HIV
10   replication, and consequently from its deleterious effects.
     The prospects of applying this methodology to human AIDS
     patients in which the antisense vectors are introduced into
     the patient to protect the patient from HIV replication and
     further progression of AIDS, represents an exciting and
15   novel strategy to combat this dreadful disease (3).

     Inhibition of HIV R.T. production in "TAR" decoy cells

     The expression of the HIV genes is regulated by the viral
20   gene product designated tat. Binding of the tat gene
     product to a specific sequence on the viral RNA, called TAR,
     is required for HIV gene expression and generation of virus.
     The tat recognition sequence was mapped to the 5' end of the
     viral RNA to encompass 60 nucleotides or less of RNA (31).
25
     A 60 base pair oligonucleotide encompassing the TAR sequence
     was chemically synthesized. (see Figure 8). A retroviral
     vector as shown in Figure 1-3 was constructed in which the
     TAR containing oligonucleotide is fused to a t-RNA gene and
30   inserted into the 3' LTR of the M-MuLV based N2 vector. A
     detailed description of the construction of this vector is
     d scribed hereinabove.

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In the experiment shown in Figure 9, clonal isolates of
CEMSS cells, an HIV-1 susceptible human T-cell line
harboring TAR - containing vectors or a control vector were
5   infected with HIV-1 at a multiplicity of infection (M.O.I.)
of about 2. The ability of the vector to efficiently
synthesize TAR and thereby inhibit the replication of HIV
was determined by measuring the secretion of virus from the
cells (determined by the appearance of reverse transcriptase
10   (R.T.) activity in the supernatant of infected cells). As
shown in Figure 9, two clonal isolates of CEMSS cells that
were characterized to harbor the control vector did support
HIV replication. This is documented by the appearance of
reverse transcriptase (R.T.) activity in the media.
15

On the other hand, in two cloned CEMSS isolates
characterized to harbor and express the TAR sequences, HIV
replication was significantly inhibited, i.e., in a range
from 90-95% inhibition.
20

Figure 10 shows the results of a separate experiment which
was conducted as described above, except that R.T. activity
was measured at intervals up to 24 days post infection. As
can be seen in Figure 10, not only is HIV replication
25   significantly inhibited as compared to the control at 14
days post infection, but the duration of the inhibitory
effect was established up to 24 days post infection.

The experiments shown in Figures 9 and 10 clearly
30   demonstrate that the synthesis of a chimeric TAR containing
transcript acts as "decoy" to bind the tat gene product
synth sized in the HIV infected cells, thereby pr venting

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the binding of tat to its physiological target, the TAR
sequence in the HIV genome. This leads to the lack of
activation of HIV gene expression and inhibition of HIV
5   replication in cultured CEMSS cells.

**Potential applications of pol III based transcriptional
units mediated via stable gene transfer**

10   The ability to synthesize high levels of specific RNA
transcripts in eucaryotic cells in a genetically stable
manner offers wide range of useful applications and a few
examples are listed below.

15   **Protection of human patients from infectious pathogens**

Antisense RNA inhibition protocols mediated via stable gene
transfer can be used to render cells resistant to the
replication of pathogens. The strategy involves the
20   introduction, via an antisense vector, of antisense
templates, i.e., a DNA molecule encoding a transcriptional
unit, which upon introduction into a cell is capable of
synthesizing an antisense RNA molecule, into the uninfected
cell to express constitutively antisense RNA specific to a
25   given pathogen. When challenged with the pathogen,
expression of its genes or any other aspect of its
replication will be inhibited and consequently pathogen
replication in the cell and spread to other cells will be
limited. The feasibility of such a strategy to inhibit the
30   replication of HTLV-I, a human retrovirus, in human T-
lymphoid cells was recently demonstrated (30) and the
experiments shown in Figures 5 and 6 demonstrated that t-RNA

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based antisense templates are effective in protecting cells from M-MuLV replication. The application of this technology to the treatment of AIDS and other pathogen caused diseases can be considered (3). It may be also possible to use antisense RNA technology to inhibit other genes which are deleterious to human patients such as oncogenies.

Generation of animals and plant breeds (transgenic) which are resistant to pathogens

Generation of transgenic animals and plants which carry effective antisense templates can be used to generate new animal and plant breeds which are resistant to a host of pathogens. Antisense RNA based strategies have been used with limited success to generate transgenic plants, in many cases achieving a level of inhibition insufficient to provide effective protection from pathogens (22).

The experiments described in the previous section have shown that pol III based promoters can be used to express in a genetically stable manner, high levels of desired RNA transcripts in the eucaryotic cell, and may be useful in the generation of animal and plant breeds carrying effective antisense templates. The same technology can be also used in a general way to inactivate specific genes in transgenic animal or plants. This can be used as a means to regulate various functions and properties of the transgenic breeds (21) or to introduce specific mutations to generate model systems for human genetic disorders (19), or to investigate consequences of gene dysfunction.

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Additional strategies of gene inhibition which require efficient RNA synthesis

5   The concept of gene inhibition via stable gene transfer was
         recently discussed in a commentary in Nature and termed
         "intracellular immunization" because it can be used to
         protect a cell from the replication of infectious pathogens
         (3). There are potentially a large number of mechanisms
10   that can be employed for this purpose (i.e., gene
         inhibition) antisense RNA inhibition being one such
         approach. Another strategy, involving the design of a
         defective regulatory protein which functions as a
         competitive inhibitor during gene expression and replication
15   of Herpes Simplex Virus (HSV), also was described (8).

         One particular application which involves the use of a
         regulatory protein which functions as a competitive
20   inhibitor to bind to an HIV protein necessary for activating
         transcription of HIV genes, involves the vector construct
         containing TAR sequences.

         In this "intracellular immunization" therapy, bone marrow
25   cells would be taken from a patient, including all the
         important haematopoietic stem cells. The stem cells will
         then be infected with the TAR-containing retroviral vector,
         in an amount which is effective to produce an amount of the
         TAR oligonucleotide decoy, such that the HIV tat protein
30   binds to the TAR decoys and is competed away from binding to
         the HIV genome, thereby inhibiting the activation of
         transcription.

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These modified stem cells will then be injected into the
patient and back to the bone marrow by methods well known to
those of ordinary skill in the art. Radiation therapy or
the administration of a cytotoxic drug to the patient may be
used to facilitate the growth of the implanted cells. This
"intracellular immunization" therapy would be useful not
only to treat HIV positive patients, but would also be
useful to prevent HIV infection.

This section describes three strategies for genes inhibition
which differ from the antisense inhibition strategy which
involves the formation of stable RNA:RNA or RNA:DNA hybrids
as described by Weintraub, et al. (37), Coleman et al. (5),
and van der Krol, et al. (22). The common denominator of
the three strategies is their dependence on the ability to
express high levels of specific RNAs in the cell, which is
the subject of this invention.

False priming

This approach is useful for the inhibition of replication of
retroviruses such as HIV, the etiological agent of AIDS.
Upon penetration of a virus into the cell, one of the
earliest events in its replication involves the reverse
transcription of the viral RNA genome into a double stranded
DNA form. This reaction is carried out by a virus specific
enzyme called reverse transcriptase (R.T.). Initiation of
reverse transcription occurs via a priming reaction in which
a cellular t-RNA bound to a specific region of the viral
genome serves as a primer. Elongation involves not only
synthesis of the DNA strand but it also involves the

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degradation of the RNA template by an RNAase H activity
which is part of or physically associated with reverse
transcriptase (36).

5

The strategy of false priming involves the synthesis of
specific primers which will initiate reverse transcription
and virion RNA degradation at various locations throughout
the viral genome, and hence lead to abrogation of the
10   replication cycle.

The advantage of false priming as compared to antisense RNA
inhibition which requires the formation of thermodynamically
stable RNA:RNA hybrids is that it: (a) does not require the
15   formation of stable hybrids; and (b) results in the
irreversible inhibition (via degradation) of the target RNA.

Gene inhibition via false priming requires the synthesis of
RNA which is complementary to the target RNA throughout its
20   3' end (to which the DNA strand is added). Consequently,
pol II based transcriptional units cannot be used to inhibit
genes via false priming because pol II transcripts contains
a stretch of poly A at their 3' end. On the other hand, pol
III based transcriptional units are uniquely suited to
25   inhibit genes via false priming because they can be used to
generate the required RNA primers. The unique mechanism of
RNA termination of pol III transcripts enables the design of
specific transcripts which are complementary throughout the
3' end to a given RNA template and therefore can act as
30   primers for reverse transcription. Briefly, pol III
terminates transcription in a run of Ts, four or more, which
is bracketed by a region of G and/or C nucleotides (9). The

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pol III transcript terminates after the second or third T. Consequently, a primer can be generated to correspond to a specific region of the retroviral genome which contains at the 3' end the sequence (G or C)$_2$AAA. The primer sequence which can be synthesized chemically, is fused to a pol III promoter at the 5' end. At the 3' end, the primer will contain an additional sequence of two or three T nucleotides, followed by several G and/or C nucleotides to regenerate a pol III transcription termination signal.

Gene inactivation via ribozymes

Haseloff and Gerlach (15) have recently shown that it is possible to cleave RNA molecules at specific sequences using artificial ribozymes. This experimental strategy can be used to inactivate specific genes by designing a transcriptional unit encoding such ribozymes.

Functional inactivation of gene regulators

Another approach which requires the efficient synthesis of specific RNA species in the cell, may be used to functionally inactivate gene activators or inhibitors and consequently inhibit or activate gene expression. For example, the HIV encoded rev gene product is a gene activator and regulates the expression of the HIV genome in the infected cell via direct binding to a specific sequence on the HIV genome called RRE (12, 24). By engineering a cell which expresses high levels of an RNA transcript containing the RRE sequence, the rev gene will bind to this RNA and therefor will not be available for binding to the

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HIV RRE sequence. Consequently, the HIV genes will not be expressed, thereby inhibiting HIV production and spread.

In a similar fashion it may be possible to use t-RNA promoter based RNA transcriptional units to inactivate the function of gene regulators which mediate their function by binding to specific DNA sequences. This can be achieved by designing a DNA template that generates an RNA transcript containing a palindrome which will fold into a double stranded structure containing the sequence corresponding to the recognition sequence of the DNA binding gene regulator. The presence of high levels of double stranded RNA forms of the recognition sequence, compared to only two copies of the actual DNA sequence in the cell, may compete effectively for the DNA binding protein.

Large scale production of proteins in eucaryotic cells

The advent of genetic engineering opened the door to the large scale production of specific proteins which in turn spawned a whole new biotechnology industry. Although bacteria are the host of choice in this process, in many cases it is necessary to use mammalian cell based production systems to obtain biologically active products. Mammalian cell based production systems are more complex, expensive and far less efficient in protein synthesis in comparison to bacterial based systems. Presently, the main strategy used to improve the production of genetically engineered proteins in mammalian cells involves the co-amplification of the corresponding gene which is fused to an amplifiable gene such as DHFR. The major limitation of this approach is that

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the amplification process requires about 8-12 months and is fraught with uncertainties, mainly due to the frequent loss of the desired gene during the amplification process.

The experiments described hereinabove offer an alternative approach for the large scale production of proteins in mammalian cells because it generates 100-10,000 fold higher levels of RNA transcripts in the cell as compared to conventionally employed pol II based transcriptional unit systems. The pol III based system can potentially produce mRNA for protein synthesis without the lengthy amplification process.

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What is claimed is:

1. A stably transformed eucaryotic cell comprising a pol III promoter and a foreign transcribable DNA, the foreign transcribable DNA being under the control of the pol III promoter.

2. A stably transformed eucaryotic cell of claim 1, wherein the foreign transcribable DNA encodes a false primer.

3. A stably transformed eucaryotic cell of claim 1, wherein the foreign transcribable DNA encodes a ribozyme.

4. A stably transformed eucaryotic cell of claim 1, wherein the foreign transcribable DNA encodes an antisense RNA.

5. A stably transformed eucaryotic cell of claim 1, wherein the foreign transcribable DNA encodes an mRNA.

6. A stably transformed eucaryotic cell of claim 1, wherein the foreign transcribable DNA encodes a polypeptide.

7. A stably transformed eucaryotic cell of claim 1, wherein the pol III promoter comprises a t-RNA or a mutant or derivative thereof.

8. A stably transformed eucaryotic cell of claim 7,

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wherein the pol III promoter comprises a human t-RNA or
a mutant or derivative thereof.

9.   A stably transformed eucaryotic cell of claim 8,
wherein the pol III promoter comprises a t-RNAi^{met} or a
mutant or derivative thereof.

10.   A stably transformed eucaryotic cell of claim 8,
wherein the pol III promoter comprises a plant pol III
promoter or a mutant or derivative thereof.

11.   A stably transformed eucaryotic cell of claim 8,
wherein the pol III promoter comprises an animal pol
III promoter or a mutant or derivative thereof.

12.   A stably transformed eucaryotic cell of claim 1,
wherein the foreign transcribable DNA encodes a
molecule which inhibits expression of a gene within the
cell.

13.   A stably transformed eucaryotic cell of claim 4,
wherein the antisense RNA comprises RNA which is
complementary of a segment to an RNA encoded by a
pathogen.

14.   A stably transformed eucaryotic cell of claim 13,
wherein the RNA encoded by pathogen comprises mRNA.

15.   A stably transformed eucaryotic cell of claim 13,
wherein the pathogen comprises the Human
Immunodeficiency Virus (HIV).

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16. A stably transformed eucaryotic cell of claim 15, wherein the foreign transcribable DNA encodes the recognition signal of the HIV gene product designated REV.

17. A stably transformed eucaryotic cell of claim 1, wherein the foreign transcribable DNA encodes an HIV regulatory sequence.

18. A stably transformed eucaryotic cell of claim 17, wherein the HIV regulatory sequence is the HIV sequence designated TAR.

19. A stably transformed eucaryotic cell of claim 1, wherein the foreign transcribable DNA encodes a recognition sequence of a regulator of gene expression which acts through binding to a DNA molecule or an RNA molecule.

20. A stably transformed eucaryotic cell of claim 1, wherein the eucaryotic cell comprises an animal cell.

21. A stably transformed eucaryotic cell of claim 20, wherein the animal cell comprises a mammalian cell.

22. A stably transformed eucaryotic cell of claim 21, wherein the mammalian cell comprises a human cell.

23. A stably transformed eucaryotic cell of claim 20, wherein the animal cell comprises a chicken cell.

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24. A stably transformed mammalian cell of claim 21,
     wherein the mammalian cell comprises a haematopoietic
     stem cell.

25. A stably transformed human cell of claim 22, wherein
     the human cell comprises a haematopoietic stem cell.

26. A stably transformed eucaryotic cell of claim 1,
     wherein the eucaryotic cell comprises a plant cell.

27. A stably transformed eucaryotic cell of claim 1,
     wherein the pol III promoter and the foreign
     transcribable DNA are present in a gene transfer
     vector.

28. A stably transformed eucaryotic cell of claim 27,
     wherein the gene transfer vector is a retroviral
     vector.

29. A stably transformed eucaryotic cell of claim 28,
     wherein the retroviral vector comprises a chimeric t-
     RNA introduced into the 3' long terminal repeat (LTR)
     region of the retroviral vector.

30. A stably transformed eucaryotic cell of claim 28,
     wherein the retroviral vector comprises the murine
     retrovirus designated M-MuLV.

31. A stably transformed eucaryotic cell of claim 28,
     wherein the retroviral vector comprises the retrovirus
     designated N2.

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32. A stably transformed eucaryotic cell of claim 28, wherein the retroviral vector comprises the retrovirus designated DCT5A.

33. A stably transformed eucaryotic cell of claim 28, wherein the retroviral vector comprises the retrovirus designated DCT5B.

34. A stably transformed eucaryotic cell of claim 28, wherein the retroviral vector comprises the retrovirus designated DCT5C.

35. A stably transformed eucaryotic cell of claim 29, wherein the chimeric t-RNA comprises a foreign DNA molecule under the control of a t-RNA termination signal, and the termination signal having removed therefrom the 3' end processing DNA sequences.

36. A stably transformed eucaryotic cell of claim 1 comprising two or more pol III promoters and two or more foreign transcribable DNAs, and each of the foreign transcribable DNAs being under the control of one of the pol III promoters.

37. A retroviral vector which comprises a chimeric t-RNA introduced into the 3' long terminal repeat (LTR) of the retroviral vector.

38. A retroviral vector of claim 36, wherein the chimeric t-RNA comprises a pol III promoter and a foreign transcribable DNA, the transcribabl DNA being under

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the control of the pol III promoter.

39. A retroviral vector of claim 38, wherein the foreign
     transcribable DNA encodes a false primer.

40. A retroviral vector of claim 38, wherein the foreign
     transcribable DNA encodes a ribozyme.

41. A retroviral vector of claim 38, wherein the foreign
     transcribable DNA encodes antisense RNA molecule.

42. A retroviral vector of claim 38, wherein the foreign
     transcribable DNA encodes an mRNA molecule.

43. A retroviral vector of claim 38, wherein the foreign
     transcribable DNA encodes a polypeptide.

44. A retroviral vector of claim 38, wherein the foreign
     transcribable DNA encodes a viral regulatory sequence.

45. A retroviral vector of claim 38, wherein the foreign
     transcribable DNA encodes an HIV regulatory sequence.

46. A retroviral vector of claim 44, wherein the HIV
     regulatory sequence is the HIV regulatory sequence
     designated TAR.

47. A retroviral vector of claim 38, wherein the pol III
     promoter comprises a t-RNA or a mutant or derivative
     thereof.

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48. A retroviral vector of claim 38, wherein the pol III promoter comprises a human t-RNA or a mutant or derivative thereof.

5

49. A retroviral vector of claim 38, wherein the pol III promoter comprises t-RNAi^{met} or a mutant or derivative thereof.

10

50. A retroviral vector of claim 38, wherein the pol III promoter comprises a plant pol III promoter or a mutant or derivative thereof.

51. A retroviral vector of claim 38, wherein the pol III promoter comprises an animal pol III promoter or a mutant or derivative thereof.

15

52. A retroviral vector of claim 38, wherein the foreign transcribable DNA encodes a molecule which inhibits expression of a gene within the cell.

20

53. A retroviral vector of claim 41, wherein the antisense RNA molecule comprises an RNA molecule which is complementary of a segment of an RNA encoded by a pathogen.

25

54. A retroviral vector of claim 53, wherein the RNA encoded by the pathogen comprises mRNA.

30

55. A retroviral vector of claim 53, wherein the pathogen comprises the Human Immunodeficiency Virus (HIV).

35

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56. A retroviral vector of claim 38, wherein the foreign transcribable DNA encodes the recognition signal of the HIV molecule designated REV.

57. A retroviral vector of claim 38, wherein the foreign transcribable DNA encodes a recognition sequence of a regulator of gene expression which acts through binding to a DNA molecule or an RNA molecule.

58. A retroviral vector of claim 38, wherein the retroviral vector comprises the murine retrovirus designated M-MuLV.

59. A retroviral vector of claim 38, wherein the retroviral vector comprises the retrovirus designated N2.

60. A retroviral vector of claim 38, wherein the retroviral vector comprises the retrovirus designated DCT5A.

61. A retroviral vector of claim 38, wherein the retroviral vector comprises the retrovirus designated DCT5B.

62. A retroviral vector of claim 38, wherein the retroviral vector comprises the retrovirus designated DCT5C.

63. A retroviral vector of claim 37, wherein the retroviral vector comprises two or more pol III promoters and two or more foreign transcribable DNAs, and each of the foreign transcribable DNAs being under the control of one of the pol III promoters.

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64. A transgenic animal comprising a stably transformed animal cell of claim 20.

65. A transgenic mammal comprising a stably transformed mammalian cell of claim 21.

66. A transgenic chicken comprising a stably transformed chicken cell of claim 23.

67. A transgenic mammal comprising a stably transformed mammalian cell of claim 24.

68. A transgenic plant comprising a stably transformed plant cell of claim 26.

69. A transgenic animal of claim 65, wherein the foreign transcribable DNA encodes an RNA molecule which is complementary to a segment of an RNA encoded by a pathogen.

70. A transgenic mammal of claim 64, wherein the foreign transcribable DNA encodes an RNA molecule which is complementary to a segment of an RNA encoded by a pathogen.

71. A transgenic plant of claim 68, wherein the foreign transcribable DNA encodes an RNA molecule which is complementary to a segment of an RNA encoded by a pathogen.

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72. A transgenic animal of claim 69, wherein the pathogen
comprises HIV.

73. A transgenic animal of claim 64, wherein the foreign
transcribable DNA encodes an HIV regulatory sequence.

74. A transgenic animal of claim 73, wherein the HIV
regulatory sequence comprises the HIV regulatory
sequence designated TAR.

75. A transgenic animal of claim 73, wherein the transgenic
animal is a mammal.

76. A gene transfer vector which comprises the retroviral
vector of claim 37, wherein the retroviral vector has
two or more pol III promoters and two or more foreign
transcribable DNAs, and each of the foreign
transcribable DNAs being under the control of one of
the pol III promoters.

77. A vaccine useful for immunizing a patient against HIV
infection which comprises an effective immunizing
amount of the retroviral vector of claim 46 and a
suitable carrier.

78. A method of producing a foreign RNA which comprises
culturing the stably transformed eucaryotic cell of
claim 1, under conditions permitting transcription of
the transcribable DNA and thereby producing the foreign
RNA.

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79. A method of claim 78, further comprising recovering the foreign RNA molecule so produced.

80. A method of claim 78, wherein the RNA molecule comprises antisense RNA.

81. A method of claim 78, wherein the RNA molecule comprises an mRNA molecule.

82. A method of producing a polypeptide comprising culturing the stably transformed eucaryotic cell of claim 1 under conditions permitting transcription of the transcribable DNA into RNA and translation of the RNA into the polypeptide and thereby producing a polypeptide.

83. A method of claim 82, further comprising recovering the polypeptide so produced.

84. A method of treating an Acquired Immunodeficiency Syndrome (AIDS) patient which comprises administering to the patient the retroviral vector of claim 46.

85. A method of treating an Acquired Immunodeficiency Syndrome (AIDS) patient which comprises administering to the patient the retroviral vector of claim 46.

86. A method of immunizing a patient against HIV infection which comprises administering to the patient an effective immunizing amount of the vaccine of claim 77.

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87. A method of intracellularly immunizing a patient
against HIV infection which comprises:

a) removing haematopoietic stem cells from the
patient;

b) infecting the removed haematopoietic stem cells
with effective amount of the retroviral vector of
claim 46; and

c) injecting back into the patient the retroviral
infected haematopoietic cells to intracellularly
immunize a patient against HIV infection.

FIGURE 1A

B.

tRNA_i^{met} 3 - 5 | 5' | ▨▨▨▨▨▨ | GCTTTTTCG
                                                CGAAAAAGC

A   B

TRANSCRIPTION

PRIMARY
TRANSCRIPT   5'——————————GCUUU-3'

5' PROCESSING

5'——————————GCUUU-3'
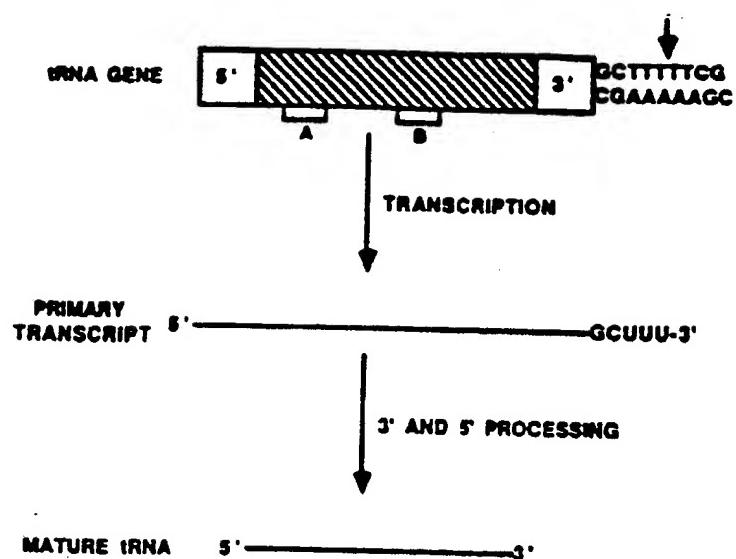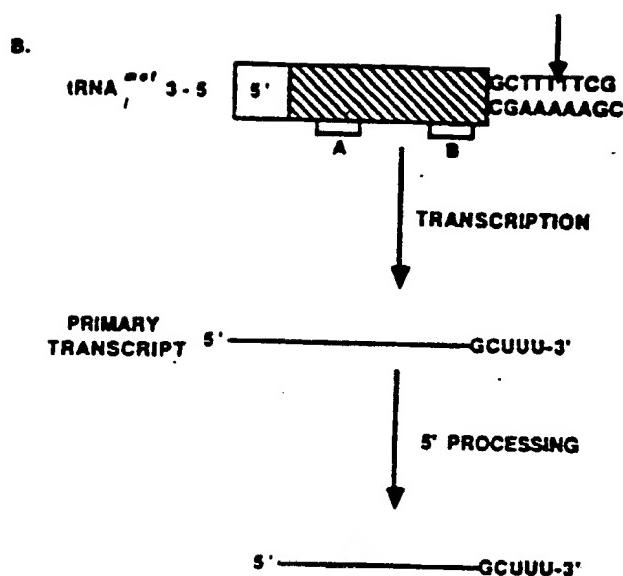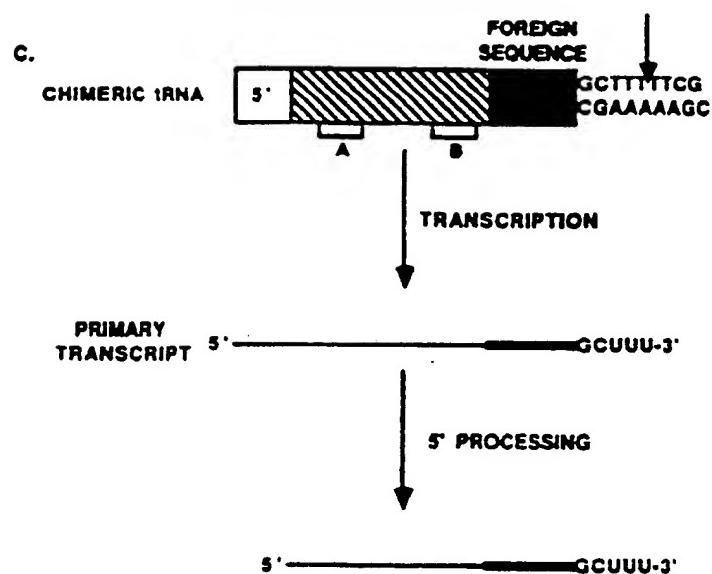
FIGURE 1B

FIGURE 1C

A.

SACII   TERMINATION   MLU I

5'-GGACACTACTTGAAGCACTCAAGGCAAGCTTTTGCTA-3'

3'-CGCCTGTGATGAACTTCGTGAGTTCCGTTCGAAAACGATGCGC-5'

B.

SACII   TERMINATION   MLU I

5'-GGCTTCCTGCCATAGGAGATGCCTAAGCCTTTTGGCA-3'

3'- CGCCGAAGGACGGTATCCTCTACGGATTCGGAAAACCGTGCGC-5'

C.

SACII   TERMINATION   MLU I

5'-GGCCAAATTGGTGGGGCTTCTGCCCGCGTTTTGGA-3'

3'- CGCCGGTTTAACCACCCCGAAGACGGGCGCAAAAACCTGCGC-5'
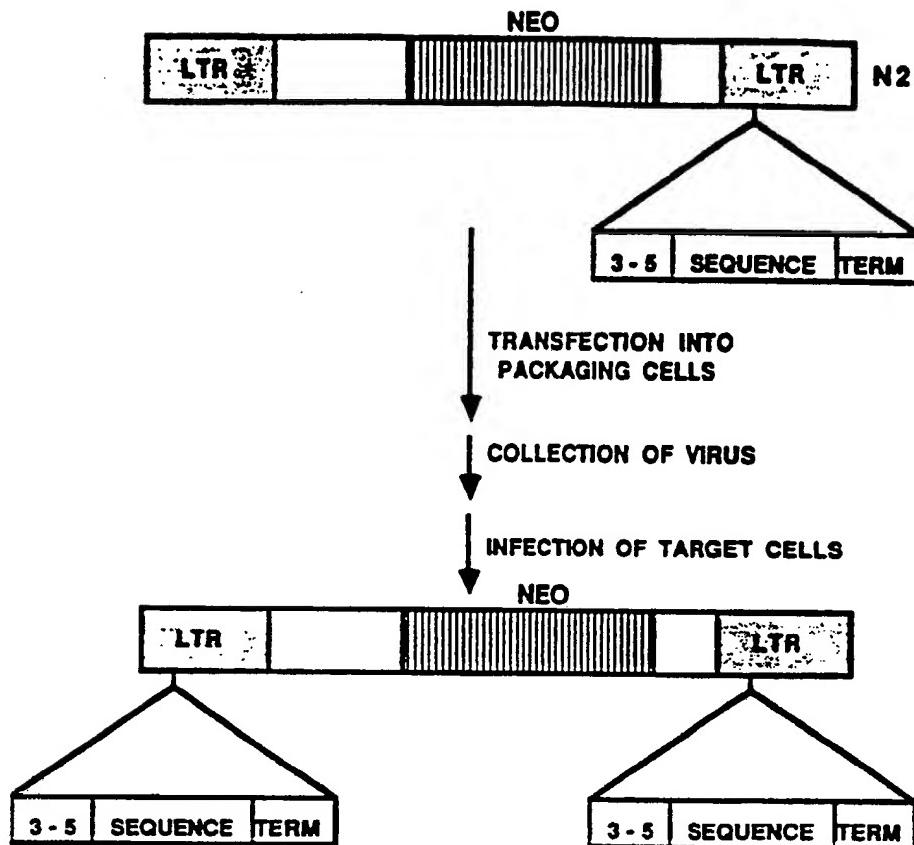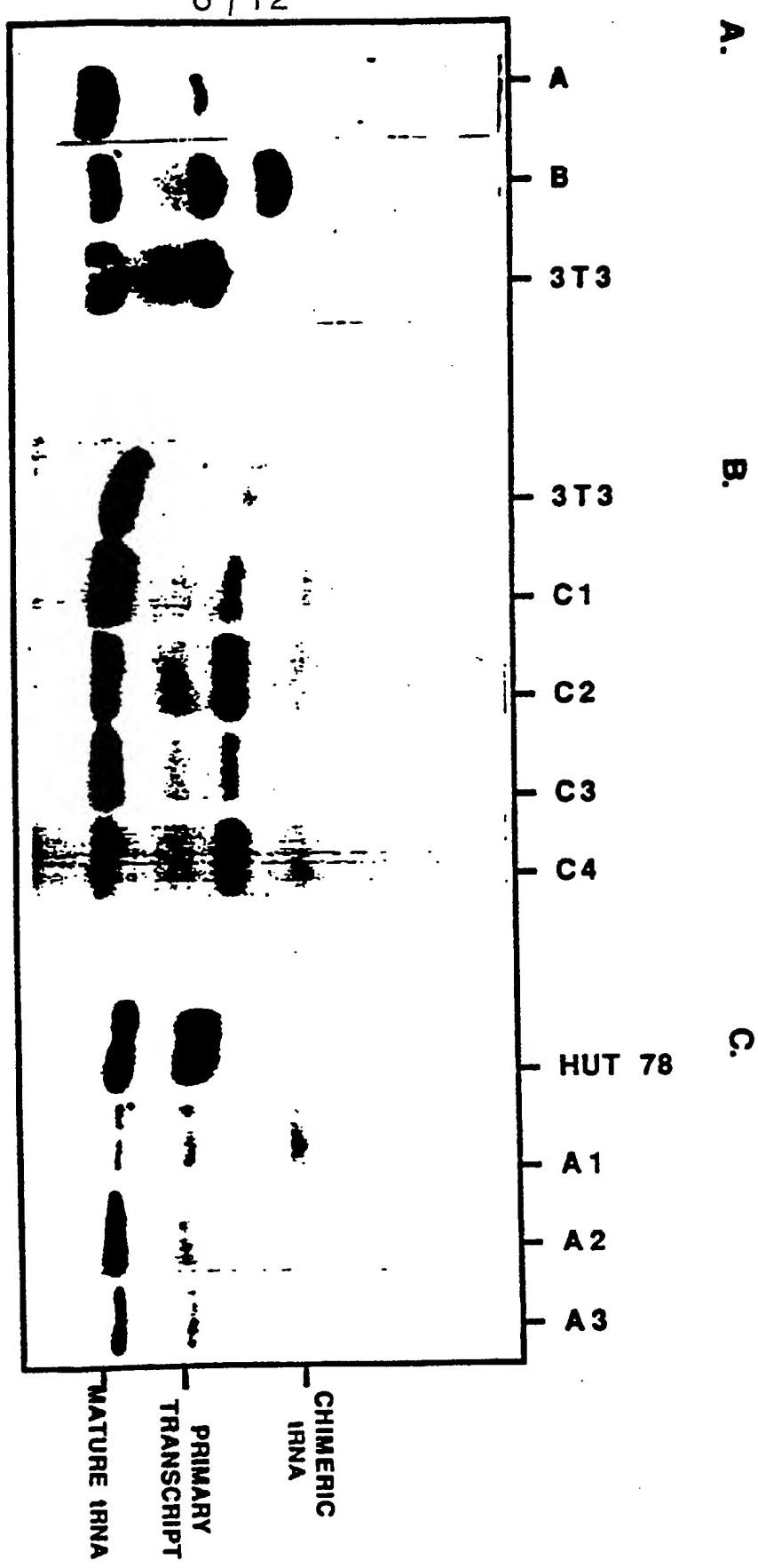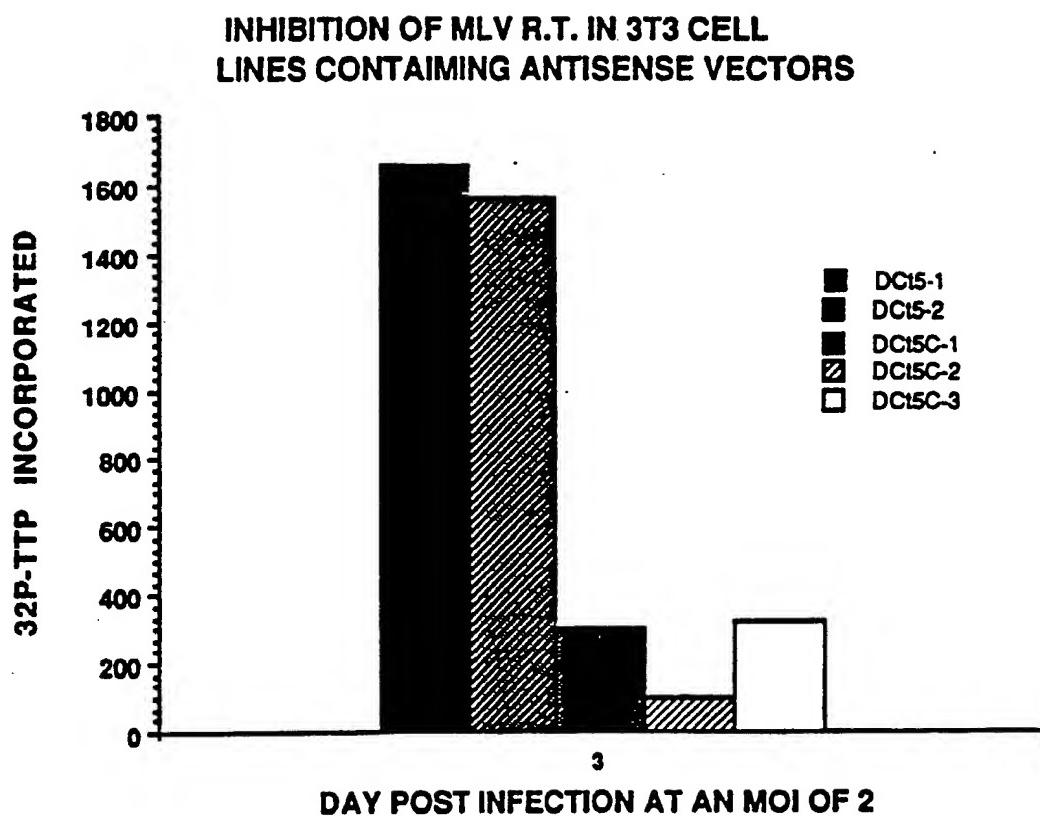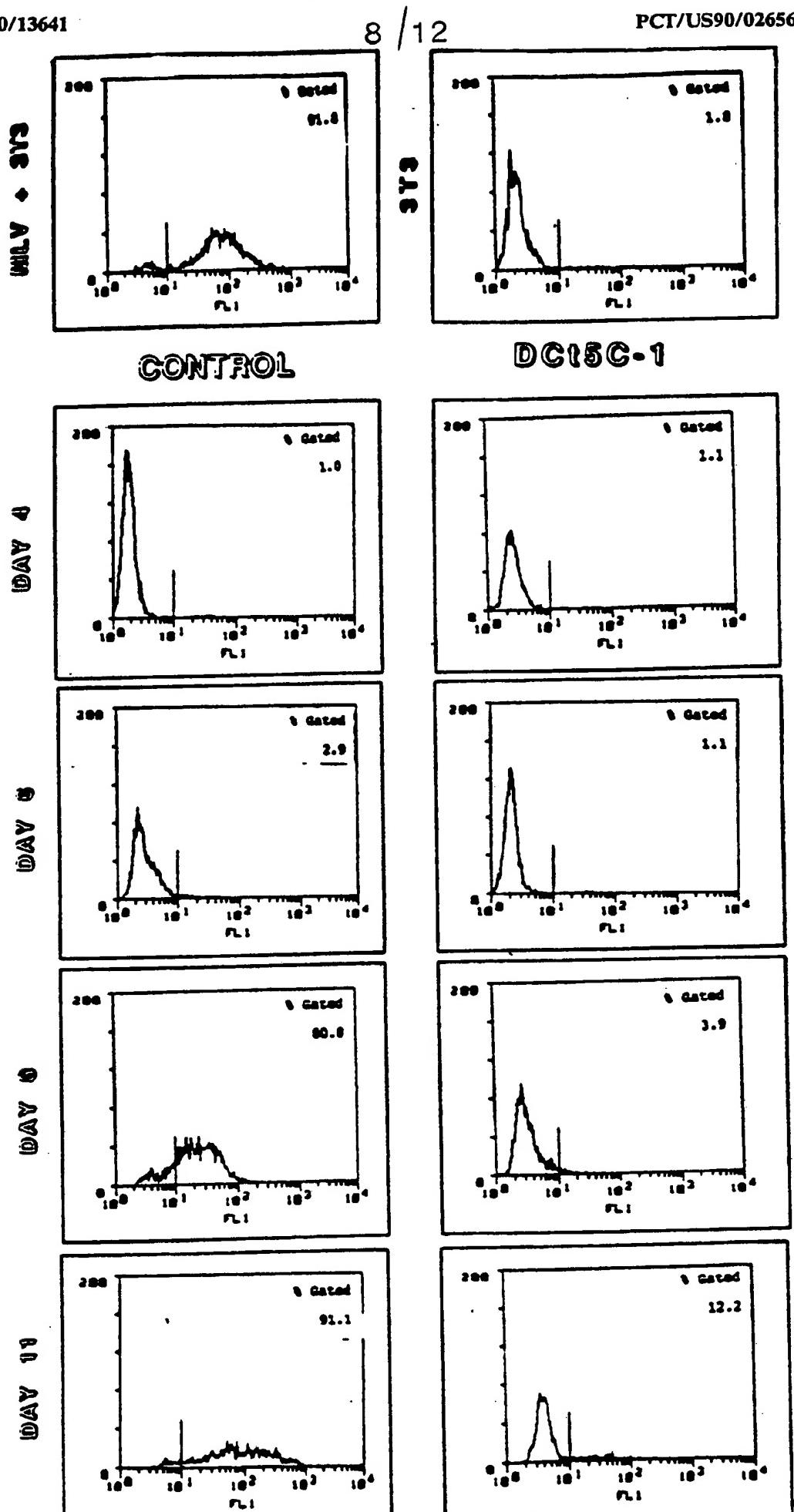
FIGURE 2

FIGURE 3

FIGURE 4
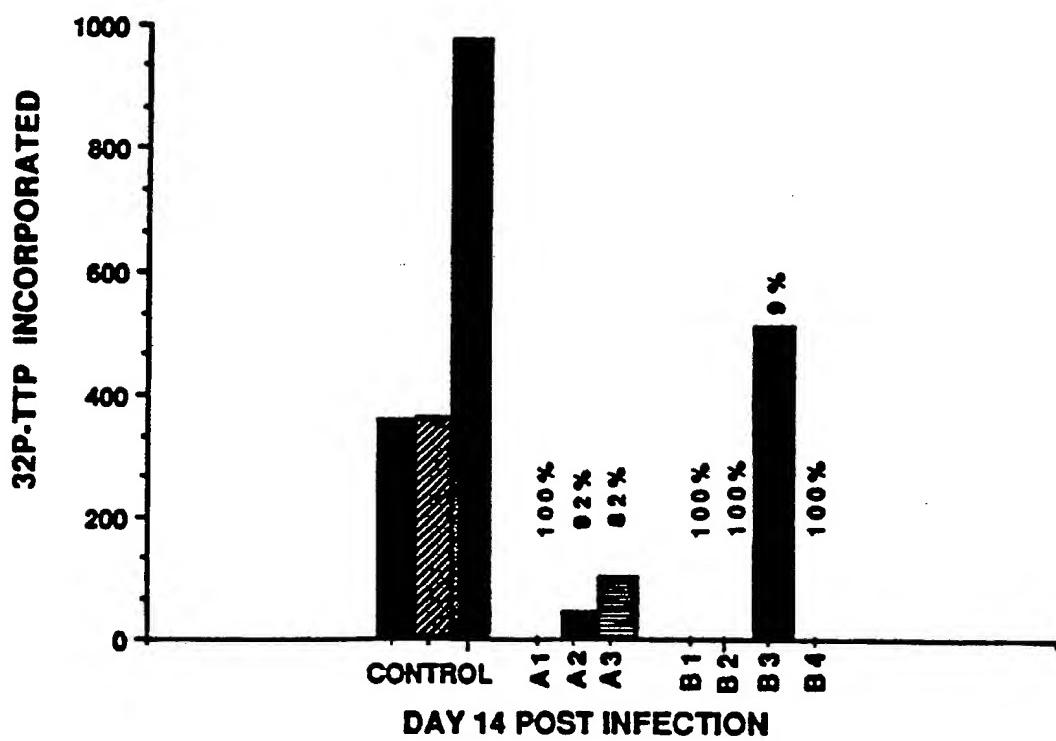
FIGURE 5

FIGURE 6

FIGURE 7

FIGURE 8

TAR SEQUENCE OLIGONUCLEOTIDE

CODING SEQUENCE

```
                                                        BGL II
        SAC II                                       ┌──────────┐
5'        GG/G GTC TCT CTG GTT AGA CCA GAT CTG AGC
```

```
                                                      BAM HI
CTG GGA GCT CTC TGG CTA ACT AGG GAA CCC AC/G
```

FIGURE 9

INHIBITION OF R.T. ACTIVITY IN
TAR "DECOY" CONTAINING CELLS
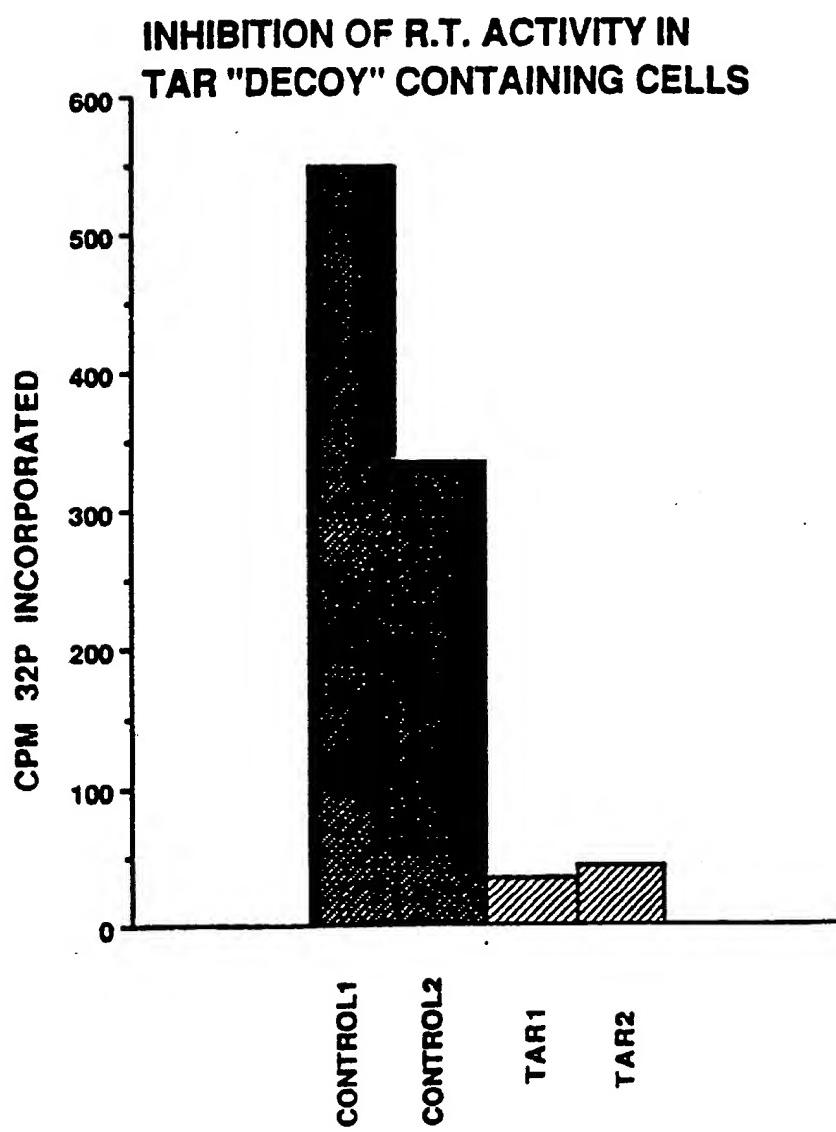
FIGURE 10

INHIBITION OF HIV-I R.T. PRODUCTION IN TAR DECOY CELLS
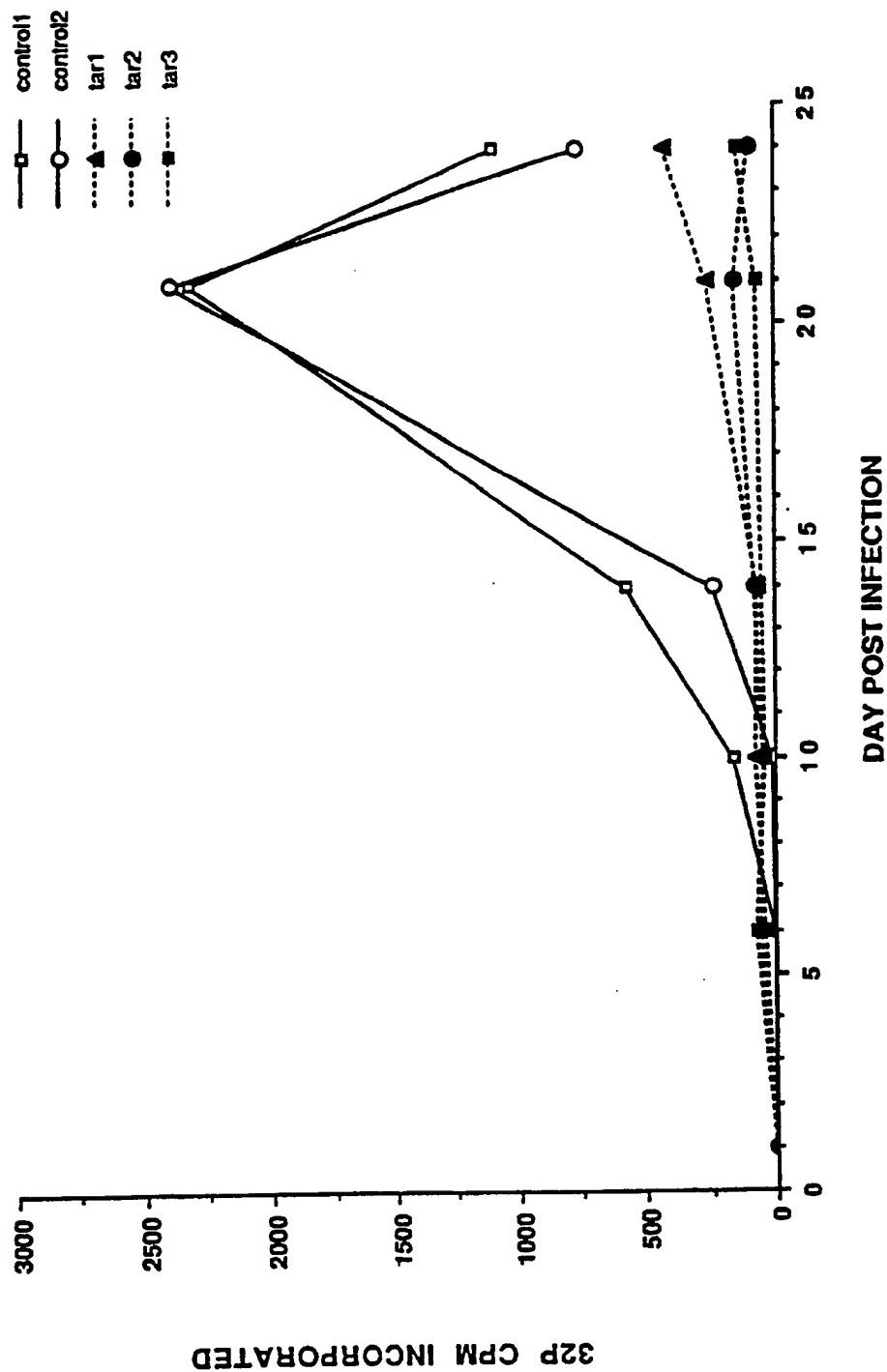
control1
control2
tar1
tar2
tar3

INTERNATIONAL SEARCH REPORT

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all) ³

According to International Patent Classification (IPC) or to both National Classification and IPC

IPC(5): C12N 15/00, 7/00, 5/00; C12P 21/00, 19/30; A61K 39/12, 37/00
U.S. CL.: 435/172.3,235,320,240.2,240.4,255,69.1,89; 424/89,93

II. FIELDS SEARCHED

Minimum Documentation Searched ⁴

Classification System	Classification Symbols
U.S.	435/172.3,235,320,240.2,240.4,255,69.1,89 424/89,93

Documentation Searched other than Minimum Documentation
to the Extent that such Documents are Included in the Fields Searched ⁶

DIALOG Databases: BIOTECH (1969-1990), CAS (1969-1990)

III. DOCUMENTS CONSIDERED TO BE RELEVANT ¹⁴

Category *	Citation of Document, ¹⁶ with indication, where appropriate, of the relevant passages ¹⁷	Relevant to Claim No. ¹⁸
	SEE ATTACHED SHEETS	

* Special categories of cited documents: ¹⁵

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

IV. CERTIFICATION

Date of the Actual Completion of the International Search ²	Date of Mailing of this International Search Report ²
29 AUGUST 1990	28 SEP 1990
International Searching Authority ¹	Signature of Authorized Officer ¹⁰
ISA/US	BETH A. BURROUS

Attachment to Form PCT/ISA/210 (second sheet)   #1

Part III. Documents considered to be relevant

Category	Citation	Claim No.
Y	Nucl. Acid. Res., Volume 11, number 6, published 1983. D. Jolly, et al. "Elements in the long terminal repeat of murine retroviruses stable transformation by thymidine kinase gene," pp. 1855-1872. see entire document.	1-67 69-70 72-87
Y	EMBO J., Volume 6, number 10, published October 1987. P. Jennings, et al. "Inhibition of SV40 replicon function · by engineered antisense RNA transcribed by RNA poly-merase III" pp. 3043-3047. see entire article.	1-67 69-70 72-87
Y	J. Virol., Volume 63, number 3, published March 1989. M. Hadzopoulou-Cladaras, et al. "The rev (trs/art) pro-tein of human immunodeficiency virus type 1 affects viral mRNA and protein expression via a cis-acting sequence in the env region" pp. 1265-1274. see entire article.	13-19 32-34 44-46 52-57 59-62 70-74 77,84-87
Y,P	Chemical Abstracts, Volume 111, no. 13, issued 1989, September 25 (Columbus, Ohio, U.S.A.). E. Gilboa, et al. "Retroviral gene transfer: applications to human therapy" see page 110247, column 1, the abstract no. 110249f, Adv. Exp. Biol. Med. 1988, 241 (Mol. Biol. Hemopoesis), 29-33 (Eng).	1-67 69-70 72-87
Y	Nature, Volume 335, published 29 September 1988. D. Baltimore "Intracellular Immunization" pp. 395-396. see entire article.	87

Attachment to Form PCT/ISA/210 (second sheet) #2

Y · Nature, Volume 335, published 29 September 1988.   1-87
A. Friedman, et al. "Expression of a truncated viral
trans-activator selectively impedes lytic infection
by its cognate virus" pp. 452-454. see entire article.

Y Nucl. Acid. Res., Volume 12, number 2, published 1984.   1-87
S. Adeniyi-Jones, et al. "Generation of long read-through
transcripts in vivo and in vitro by deletion of 3' termin-
ation and processing sequences in the human tRNA (i)(met)
gene" pp. 1101-1115. see entire article.   /

Y Cell, Volume 43, published December 1985. J. Tobian,   1-87
et al. "tRNA nuclear transport: defining the critical re-
gions of human tRNA (i)(met) by point mutagenesis"
pp. 415-422. see entire article.

Y J. Virol., Volume 63, number 2, published February 1989. 1-87
T. von Ruden, et al. "Inhibition of human T-cell leukemia
virus type I replication in primary human T cells that
express antisense RNA" pp. 677-682. see entire article.

Y Nature, Volume 323, published 25 September 1986.   77,
J. Zarling, et al. "T-cell responses to human AIDS   84-86
virus in macaques immunized with recombinant virus"
pp. 344-346. see entire article.

Y US, A, 4,497,796 (SALSER ET AL.) 05 February 1985.   64-67
See entire document.   69,70
72-75

Y Cold Spring Harbor Sym. on Mol. Biol., Volume LI.   64-67
published 1986. A. Miller, et al. "Transfer of genes into   69,70
human somatic cells using retrovirus vectors" pp. 1013- 72-76
1019. see entire article.

Attachment to Form PCT/ISA/210 (second sheet) #3

Y   EMBO J., Volume 6, number 2, published February 1987.   68,71
     N. Takamatsu, et al. "Expression of bacterial chloram-
     phenicol acetyltransferase gene in tobacco plants
     mediated by TMV-RNA" pp. 307-311. see entire article.